Form PTO 1390 (REV 5-93) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER P50836
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED / ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) 09/786839
INTERNATIONAL APPLICATION NO. PCT/US99/20957	INTERNATIONAL FILING DATE 15 September 1999	PRIORITY DATE CLAIMED 18 September 1998
TITLE OF INVENTION CXCR2 INHIBITORS AND PMN ADHESION AND T-CELL CHEMOTAXIS		
APPLICANT(S) FOR DO/EO/US John R. White		

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1 [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. [X] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] has been transmitted by the International Bureau.
   c. [X] is not required, as the application was filed in the United States Receiving Office (RO/US).

6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. [ ] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.

8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S. C. 371(c)(3)).

9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. [X] An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.

12. [X] An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.

13. [ ] A FIRST preliminary amendment.

14. [ ] A SECOND or SUBSEQUENT preliminary amendment.

15. [ ] Please amend the specification by inserting before the first line the sentence: This is a 371 of International Application **PCT/US99/20957**, filed **September 15, 1999**, which claims benefit from the following Provisional Application **60/101,021**, filed **September 18, 1998**.

16. [ ] A substitute specification.

17. [ ] A change of power of attorney and/or address letter.

18. [x] An Abstract on a separate sheet of paper.

19. [ ] Other items or information:

US APPLICATION NO. (if known see 37 CFR 1.50)	INTERNATIONAL APPLICATION NO.	ATTORNEYS DOCKET NO.
09/786839	PCT/US99/20957	P50836

20. [X] The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 C.F.R. 1.492(a)(1)-(5)):		
Search Report has been prepared by the EPO or JPO **$860.00**		
International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) **$690.00**		
No International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) **$710.00**		
Neither International Preliminary Examination Fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....**$1,000.00**		
International Preliminary Examination Fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).....**$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$860.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	**$0.00**	

Claims	Number Filed	Number Extra	Rate		
Total claims	**6 - 20 =**	**0**	**0 x $18.00**	**$0.00**	
Independent claims	**2 - 3 =**	**0**	**0 x $80.00**	**$0.00**	
Multiple dependent claims (if applicable)			**+ $270.00**	**$0.00**	
			TOTAL OF ABOVE CALCULATIONS =	**$0.00**	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).				**$**	
			SUBTOTAL =	**$0.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)) +				**$**	
			TOTAL NATIONAL FEE =	**$860.00**	
				Amount to be refunded	$
				charged	$

a. ☐ A check in the amount of $____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. 19-2570 in the amount of **$860.00** to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-2570 . A duplicate copy of this sheet is enclosed.

d. ☒ General Authorization to charge any and all fees under 37 CFR 1.16 or 1.17, including petitions for extension of time relating to this application (37 CFR 1.136 (a)(3)).

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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document3

CXCR2 INHIBITORS AND PMN ADHESION AND T-CELL CHEMOTAXIS

Field of The Invention

The present invention relates to the novel use of CXCR2 inhibitors for the treatment of diseases mediated thereby.

Background of the Invention

The recruitment of inflammatory cells into sites of tissue damage is a normal physiological response designed to fight infection, remove damaged cells, and stimulate healing. However, the excessive recruitment of such cells often exacerbates tissue damage, slows healing and in some cases leads to host death. Therefore, inhibition of inflammatory cell recruitment may be an appropriate therapeutic strategy in a number of inflammatory diseases, such as reperfusion injury, arthritis, asthma atherosclerosis and inflammatory bowel disease.

Summary of the Invention

The present invention is to a method of inhibiting, or blocking, the binding of human neutrophils to activated endothelial cells in a patient in need thereof, which method comprises administering to said patient an effective amount of a compound which binds the CXCR2 receptor.

Preferably, the compound will also bind to other chemokine receptors, such as the CXCR1 receptor.

Another aspect of the present invention is a method of inhibiting or blocking T-cell mediated chemotaxis in a patient in need thereof, which method comprises adminstering to said patient an effective amount of a compound which binds the CXCR2 receptor.

Brief Description of the Drawings

Figure 1 shows a control experiment. in which Human Umbilical Cord EndothelialCells (HUVEC) are treated with IL-1β and IL-8 in the absence of CXCR2 antagonist. IL-1β and IL-8 stimulate neutrophils to roll along the endothelial cell surface and to firmly adhere to these cells.

Figure 2 demonstrates that administration of compound 1, N-[2-Hydroxy-4-cyanophenyl]-N'-[2-bromophenyl] urea blocks the firm adhesion of neutrophils to the cell surface of HUVEC but does not interfere with rolling

of PMNs on HUVEC.

Figure 3 shows the dose dependent inhibition of PMNs binding to activated HUVEC by Compound 1. Each point represents the percent of cells (compared with control) which have bound to the endothelial cells at the 4 min time mark at each of the different concentrations of Compound 1.

Figure 4 shows the dose dependent inhibition of T-cell mediated migration by compound II, N-(2-Hydroxy-4-nitrophenyl]-N'-(2-bromophenyl)urea when T-cells were stimulated to migrate to IL-8 or Groα but not to a control chemokine MCP-l.

Detailed Description of the Invention

The recruitment of neutrophils from post-capillary venules depends initially upon the rolling of neutrophils via the interaction of neutrophils expressed sLex with endothelial cells expressed E-selectin. This is followed by attachment through the up-regulation of the adhesion molecules CD11b/CD18 (Mac-1), and diapedesis via a heptotactic gradient of IL-8 (Rot, et al., *J. Leukoc. Biol.* **59**, 39-44 (1996)). The mechanism for PMN attachment to endothelial cells is not completely understood, but may well involve the up-regulation of CD11b/CD18 on neutrophils (Detmers. et al., *J. Exp. Med.* **171**, 1155-1162 (1990)). T-cells also respond to the chemokines IL-8 and Groα and migrate to these two factors in a similar manner as neutrophils. The recruitment of T-cells to sites of antigen presentation such as can be found in arthritic joints of patients suffering from rheumatoid arthritis. is thought to be essential for the continuation of the inflammatory process.

IL-8 and Groα are members of the super family of proinflammatory proteins known as chemokines, which are approximately 8 kD in size. In human neutrophils and T-cells, IL-8 binds with similar affinity to two distinct 7TMRs, CXCR1 (Holmes et al., *Science* **253**, 1278-1280 (1991)) and CXCR2 (Murphy, et al., *Science* **253**, 1280-1283 (1991)), whereas closely related chemokines containing a common amino-terminal Glu^4-Leu^5-Arg^6 (ELR) amino acid sequence, including GRO-α, NAP-2 and ENA-78, bind only to CXCR2 (Hebert et al., *J. Biol. Chem.* **266**, 18989-18994 (1991)). Both CXCR1 and CXCR2 are present on the surface of human neutrophils and a subset of T-cells (see Holmes, et al., *Supra*; Murphy et al., *Supra*; Chuntharapai et al., *J. Immunol.* **153**, 5682-5688 (1994) and Xu et al., *J. Leukoc. Biol.* **57**, 335-342 (1995)). In human neutrophils it is unclear whether attachment to

endothelial cells is mediated by one or both receptors. In addition, it is unclear which of these two receptors expressed on human T-cells are responsible for mediating T-cell chemotaxis.

One aspect of the present invention therefore, is to a method of inhibiting, or blocking, the binding or attachment, of human neutrophils to activated endothelial cells in a patient in need thereof, which method comprises administering to said patient an effective amount of a compound which binds the CXCR2 receptor. Another aspect of the present invention, is a method of inhibiting or blocking T-cell mediated movement or chemotaxis by administering to a patient in need of this treatment a compound which binds the CXCR2 receptor. Preferably, the compound will also bind to other chemokine receptors, such as the CXCR1 receptor.

Suitable CXCR2 inhibitors which are useful in the present invention include, but are not limited to those compounds disclosed in US Patent No. 5,684,032 ; WO 96/25157 ; US Patent No. 5,780,483 ; WO 97/35572 ; WO 97/49286; WO 97/49399; WO 97/49680; WO 97/49287; WO 98/07418 ; WO 97/49400 ; WO 98/05329 ; WO 98/05317 ; WO 98/05328 ; WO 98/06398 ; WO 98/06397 ; WO 98/06399 ; WO 98/06262 ; WO 98/06701 ; WO 98/ 32439 ; and WO 98/ 32438 ; Attorney Docket No.: P50708, PCT US98/ , filed ____; and Attorney Docket No.: P50709, PCT US98/----, filed ---/ / 98

BIOLOGICAL METHODS

HUVEC preparation and activation

HUVECs at passage 2 or 3 were grown for 24 to 48 hours in glass capillary tubes until confluent in EGM culture medium (Clonetics) containing 10% FBS. The endothelial cells were then activated just prior to the assay for 1hour with 10ng/ml IL-1, washed with EGM and then incubated with IL-8 (100ng/ml) or PAF (10nM) for 3 hrs at 37°.

Neutrophil isolation

Neutrophils were isolated from human peripheral blood. Briefly, blood was collected into citrate (ACD) anti-coagulant tubes (Becton Dickinson), diluted 1:2 at room temperature in sterile buffered HBSS w/o Ca^{++} & Mg^{++} (pH 7.0) with HEPES (20mM) (Fisher Scientific) then adding 5% Dextran T500 (Pharmacia Biotech) to the blood for a 1% final concentration. The blood/HBSS was then incubated at 4 ^{o}C for 45 minutes to allow the RBCs to settle out of suspension. The remaining RBCs in suspension were lysed in cold water for 10-15 seconds and then the leukocyte

suspension was mixed 10 part HBSS with 1 part water, spun down and resuspended at a concentration of $6x10^6$ cells/ml in HBSS w/o Ca^{++} & Mg^{++} (pH 7.0). The cell suspension was then underlayed with Histopaque 1077 and Histopaque 1117 (Sigma), and centrifuged at 2,300 RPM for 30 min at room temperature. neutrophils were collected from the Histopaque 1117/1077 interface. The cells were then resuspended at a concentration of $6x10^6$ cells/ml in HBSS Then, the cells were treated with Compound 1 (or not) and incubated on ice for 15-20 minutes. Just before adding the neutrophils for the loop assay they were diluted to $3x10^6$ cells/ml in DMEM with HEPES at 20mM.

T-cell Isolation

Peripheral blood mononuclear cells were harvested from individuals that had been sensitized with Tetanus Toxin 7 days prior to the blood draw. PBLs were separated over Ficol, and washed in PBS (x2) prior to adding the cells (1 x 106 cells/ml, RPMI1640 + 5% Autologous human serum) to T75 flasks with 5 ng of Tetanus Toxin. After 5 days of culture non-adherent cells were removed and washed 2x in PBS. These cells were then used for the chemotaxis assay. The chemotaxis assay was carried out as previously described (White et. al. J. Biol. Chem 273:10095 (1998)). After chemotaxis for 5 hrs in which chemokine was placed in the bottom chamber along with a suitable concentration of compound II, N-(2-Hydroxy-4-nitrophenyl]-N'-(2-bromophenyl)urea. The migrated cells were enumerated by staining the separation membrane DiffQuick and counting the number of T-cells which had migrated to the stimulus.

Effect of Compound 1 on selectin/integrin mediated neutrophil rolling and arrest on IL-8 pretreated, IL-1 activated HUVEC

The analysis of Compound 1: N-[2-Hydroxy-4-cyanophenyl]-N'-[2-bromophenyl] urea was performed in triplicate in DMEM medium containing 1% human serum buffered with 20mM HEPES. Untreated human neutrophils were isolated and handled as previously indicated and infused into the shear system loop for recirculation through the capillary tube lined with confluent cultures of HUVEC treated with IL-1 and IL-8 or PAF as indicated above. Neutrophils were pretreated (or not) with compound 1 antagonist at 300, 150, 50 and 10nM for 15-20 minutes before infusion into the shear assay.

General in vitro shear assay

After activation, the HUVEC containing capillary tubes were connected to the assay tubing to form a closed loop in which medium and cells could be recirculated; the tube was then mounted on the inverted microscope stage. Using a variable speed peristaltic pump, flow was regulated to simulate in vivo shear conditions (1.8 - 2 dynes/cm^2) (Bargatze, et al., J. Immunol. **152**:581 (1994)). Isolated human neutrophils were infused into the system at a 3x10^6 cell/ml in sterile HEPES buffered (20mM) HBSS (pH 7.0) plus 1% human serum. Rolling was established and the adhesive interactions were continuously monitored for the duration of the experiment while being videotaped for off-line analysis.

Analyses of neutrophil rolling

The number of neutrophils interacting, both binding and rolling, with activated HUVECs was quantified at 1 min intervals using NIH IMAGE software, Montana ImmunoTech Inc. macros and an Apple Computer PowerMac 7100-66. Interacting neutrophils were quantified, within 350 µm (horizontal) by 250 µm (vertical) video-microscopic fields.

Results

Data from three replicate experiments show that Compound 1, after a 15 - 20 min preincubation with neutrophils inhibits IL-8 enhanced adhesion to IL-1b activated HUVECs over a 8 minute time interval. Compound 1 did not inhibit rolling of neutrophils over the HUVEC cell surface (Figures 1 and 2). In addition, Compound 1 dose dependently inhibited neutrophil binding to HUVEC (Fig 3) with an IC50 = 20 nM. When PAF (Platelet Activating Factor) was substituted for IL-8 in the shear assay, Compound 1 at a concentration of 300 nM failed to inhibit binding of PMN's to the HUVEC cells, thus indicating that Compound 1 is a specific inhibitor of IL-8 induced function. Data also indicated that compound II was capable of inhibiting both IL-8 and Groα mediated T-cell chemotaxis but was not able to inhibit a related chemokine MCP-1 (Figure 4).

All publications, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference as if each individual publication were specifically and individually indicated to be incorporated by reference herein as though fully set forth.

The above description fully discloses the invention including preferred embodiments thereof. Modifications and improvements of the embodiments specifically disclosed herein are within the scope of the following claims. Without further elaboration, it is believed that one skilled in the are can, using the preceding description, utilize the present invention to its fullest extent. Therefore, the Examples herein are to be construed as merely illustrative and not a limitation of the scope of the present invention in any way. The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows.

What is Claimed is:

1. A method of inhibiting or blocking the binding of human neutrophils to activated endothelial cells in a patient in need thereof, which method comprises administering to said patient an effective amount of a compound which binds the CXCR2 receptor.

2. The method according to claim 1 wherein the compound is N-[2-Hydroxy-4-cyanophenyl]-N'-[2-bromophenyl] urea, or N-(2-Hydroxy-4-nitrophenyl]-N'-(2-bromophenyl)urea or a pharmaceutically acceptable salt thereof.

3. The method according to claim 1 which further comprises a compound which also binds to the CXCR1 receptor.

4. A method of inhibiting or blocking T-cell mediated chemotaxis in a patient in need thereof, which method comprises to said patient an effective amount of a compound which binds the CXCR2 receptor.

5. The method according to claim 4 wherein the compound is N-[2-Hydroxy-4-cyanophenyl]-N'-[2-bromophenyl] urea, or N-(2-Hydroxy-4-nitrophenyl]-N'-(2-bromophenyl)urea or a pharmaceutically acceptable salt thereof.

6. The method according to claim 4 which further comprises a compound which also binds to the CXCR1 receptor.

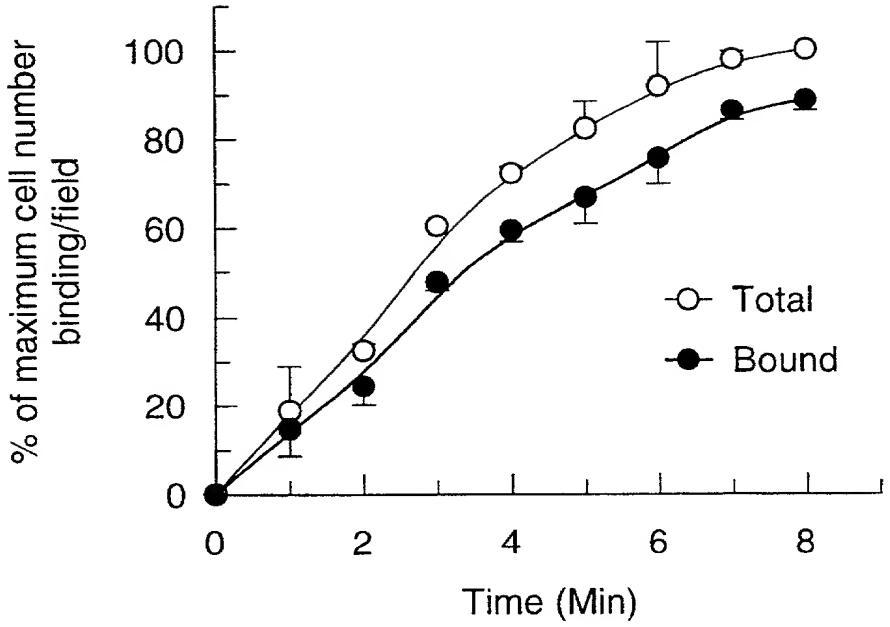
% of maximum cell number binding/field
100
80
60
40
20
0
0
2
4
6
8
Time (Min)
Total
Bound

FIGURE 1

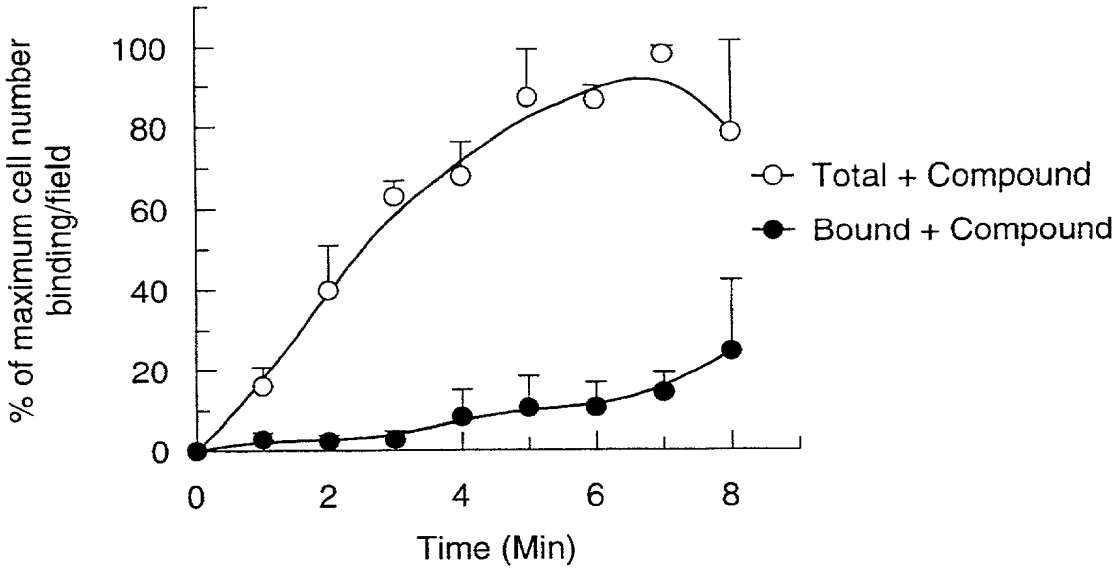

FIGURE 2

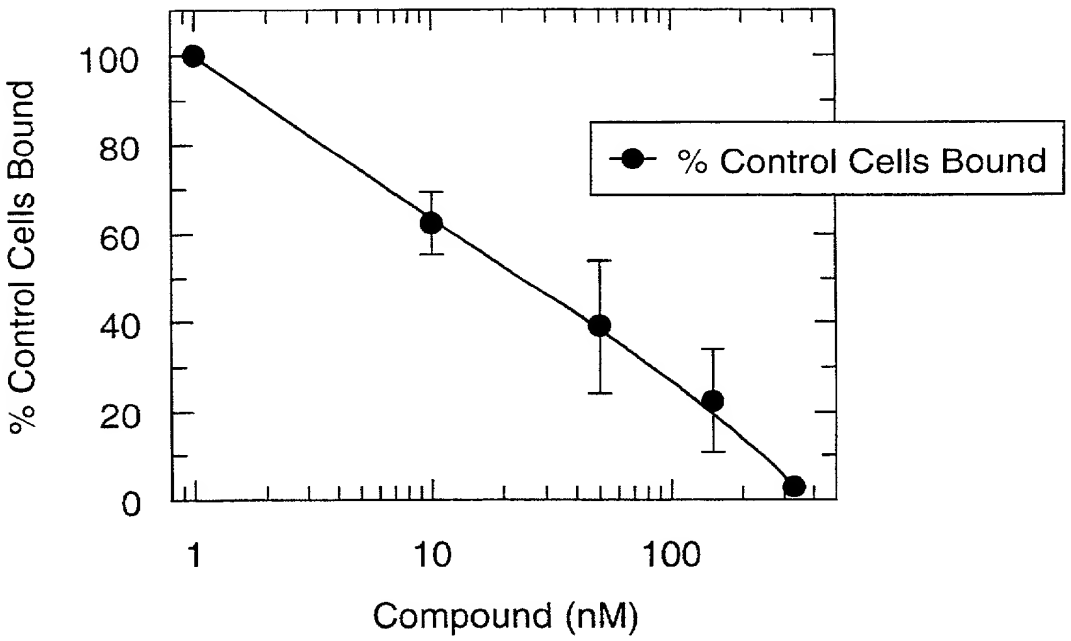
100
80
60
40
20
0
% Control Cells Bound
% Control Cells Bound
1
10
100
Compound (nM)

FIGURE 3

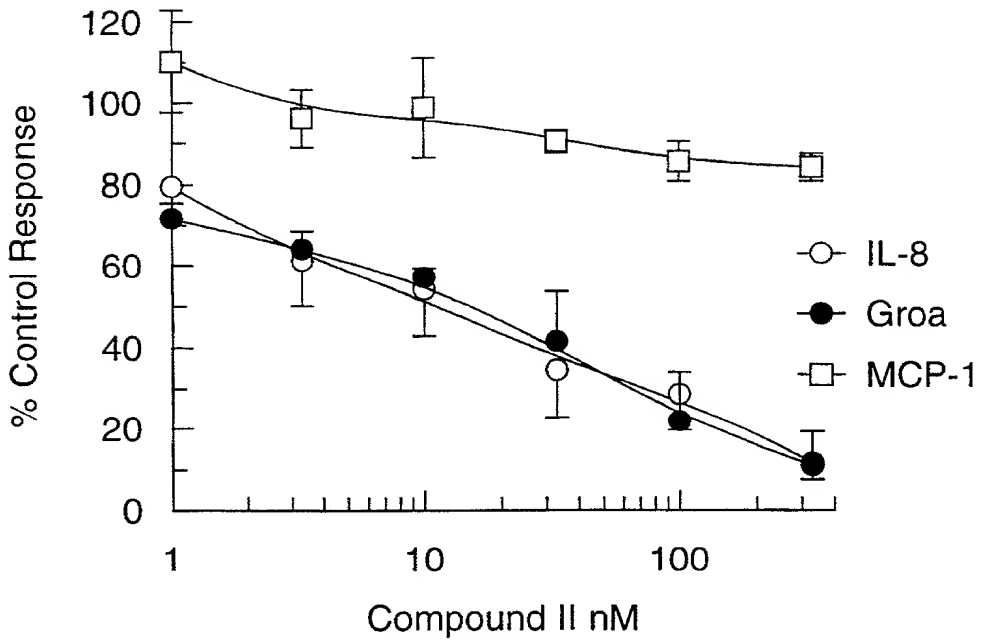
120
100
80
60
40
20
0
% Control Response
1
10
100
Compound II nM
IL-8
Groa
MCP-1

FIGURE 4

Docket No.: P50836

DECLARATION AND POWER OF ATTORNEY

Use this declaration when all of the attorneys are within SB and correspondence is addressed to 709 Swedeland Road, etc.

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

"CXCR2 INHIBITORS AND PMN ADHESION AND T-CELL CHEMOTAXIS"

the specification of which (check one)
[ ] is attached hereto.
[X] was filed on **18 September 1999** as Serial No. **PCT/US99/20957**
and was amended on (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below any foreign application for patent or Inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Number	Country	Filing Date	Priority Claimed

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below.

Application Number	Filing Date
60/101,021	**18 September 1999**

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or

PCT International application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

Serial No.	Filing Date	Status

I hereby appoint the practitioners associated with the Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and direct that all correspondence be addressed to that Customer Number:

Customer Number 20462.

Address all correspondence and telephone calls to **William R. Majarian**, SmithKline Beecham Corporation, Corporate Intellectual Property-U.S., UW2220, P.O. Box 1539, King of Prussia, Pennsylvania 19406-0939, whose telephone number is **610-270-5968**.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full Name of Inventor: **John R. White**

Inventor's Signature: _____ Date: 15-Feb-01

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